Micro-PIXE-RBS methods highlighting the influence of phosphorus on the in vitro bioactivity of sol-gel derived glass particles in the SiO2-CaO-P2O5 system

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Micro-PIXE-RBS methods highlighting the influence of phosphorus on the *in vitro* bio-activity of sol-gel derived glass particles in the SiO_2-CaO-P_2O_5 system

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Abstract

Ion beam analysis methods were used to characterize the interface of bioactive glasses with surrounding biological fluids. Glass particles in the SiO_2-CaO and SiO_2-CaO-P_2O_5 compositions were elaborated by sol-gel processing and soaked in biological fluids for periods up to 4 days. The surface changes were characterized using Particle Induced X-ray Emission (PIXE) associated to Rutherford Backscattering Spectroscopy (RBS), which are efficient methods for multielemental analysis and accurate trace elements quantification. Elemental maps of major and trace elements were obtained at a micrometer scale and revealed the bone bonding ability of the materials. Local measurements of elemental concentrations were per-

formed at the 10^{-6}g/g level. Glass particles are quickly coated with a thin calcium phosphate-rich layer containing traces of magnesium. After a few days, SiO_2-CaO-P_2O_5 glass particles are entirely changed into calcium phosphates, whereas SiO_2-CaO particles exhibit a different behavior: the previously Ca-P enriched periphery has been dissolved and glass particles consist of a silicate network. Calculation of the Ca/P atomic ratios at the glass/biological fluids interface provides us with an explanation for this: an enduring apatitic phase seems to be formed at the periphery of SiO_2-CaO-P_2O_5 glass particles. Presence of phosphorus in the glass matrix thus has an influence on the amplitude and the kinetics of reaction of the bioactivity process. It might result in an improved chemical bond with living tissues.

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Keywords: PIXE-RBS methods; biomaterials; bioactive glass; sol-gel, biomineralization.

Introduction

Bioactive glasses possess the ability to bond with bony tissues: as new generation biomaterials, they actively contribute to the healing process and can stimulate regeneration of living tissues through a direct control over genes [1, 2]. The singular properties of bioactive glasses make them particularly interesting for filling bony defects, repairing damaged tissues and struggling against troubles due to osteoporosis. In contact with body fluids, bioactive glasses induce a series of physico-chemical reactions leading to the formation of an interfacial calcium phosphate-rich layer. This layer then progressively crystallizes into a bone-like apatitic mineral [3]. The bioactivity process deeply depends on the composition and the texture of the glass. Optimizing both the material reactivity and its biointegration properties requires the collection of reliable quantitative information regarding the physico-chemical reactions occurring at the interface as well as the ionic exchanges at the surface of the glass.

For this purpose, bioactive glasses in the SiO_2–CaO and SiO_2–CaO–P_2O_5 systems were elaborated using the sol-gel method, which permits the synthesis of materials with

higher purity and homogeneity at low processing temperature [4]. Samples of gel-glass powder were immersed in biological fluids for varying periods. Analyses of major, minor and trace elements present at the biomaterial/biological fluids interface were performed by particle-induced X-ray emission (PIXE) associated to Rutherford backscattering spectroscopy (RBS). Obtaining PIXE elemental maps at a micrometer scale permits the complete follow-up of the calcium phosphate layer formation along with accurate major and trace element quantification. It allows important evaluation for the *in vitro* bioactivity.

Materials and methods

Preparation of the bioactive glass samples

Gel-glass powders containing 75wt% SiO_2–25wt% CaO, named B75, and 67.5wt % SiO_2–25wt% CaO–7.5wt% P_2O_5, named B67.5, were prepared using the sol-gel process. Tetraethylorthosilicate ($Si(OC_2H_5)_4$), triethylphosphate ($PO(OC_2H_5)_3$) and calcium nitrate $Ca(NO_3)_2$, $4H_2O$ were mixed in a solution of ethanol in presence of water and HCl. The prepared sols were then transferred to an oven at 60°C for gelification and aging. Four hours later, the obtained gels were dried at 125°C for 24 hours, then finally grinded to powder and heated at 700°C for 24 hours. The final surface area of the glasses was measured by nitrogen sorption analyses and was found to be 30 and 112 m^2/g for B75 and B67.5 respectively.

In vitro assays

10 mg of gel-glass powders were soaked at 37°C for 1, 6 h and 1, 2, 3, 4 days in a standard Dulbecco's Modified Eagle Medium (DMEM, Biochrom AG, Germany), which composition is almost equal to human plasma. In addition, because of the high reactivity of B75 glass particles, *in vitro* assays were conducted on B75 particles at very short times: 15 and 30 minutes soaking in DMEM. For the whole lot of B75 and B67.5 samples, the surface area to DMEM volume ratio was fixed at 500 cm^{-1}. After interaction, the samples were removed from the solution, air dried and embedded in resin (AGAR, Essex, England). Before

characterization, the glass particles were cut into thin sections of 1 micrometer nominal thickness using a Leica EM UC6 Ultramicrotome, and laid out on 50 mesh copper grids. The grids were then placed on a Mylar film with a hole of 3 mm in the centre.

PIXE-RBS analysis

Analyses of the glass/biological fluids interface were carried out using nuclear microprobes at the CENBG (Centre d'Études Nucléaires de Bordeaux-Gradignan, France). For PIXE-RBS analyses, we chose proton scanning micro-beam of 1.5 MeV energy and 50 pA in intensity. Such settings resulted in higher ionization cross-sections and an increased sensitivity for the micro-analysis of bioactive glasses composed of light elements ($Z \leq 20$). The beam diameter was nearly 1 micrometer. An 80 mm^2 Si(Li) detector was used for X-ray detection, orientated at 135° with respect to the incident beam axis and equipped with a beryllium window 12 μm thick. PIXE spectra were treated with the software package GUPIX [5]. Relating to RBS, a silicon particle detector placed at 135° from the incident beam axis provided us with the number of protons that interacted with the sample. Data were treated with the SIMNRA code [6].

Results

For each immersion time, multielemental maps were recorded at the interface of the glass particles with the surrounding biological fluids. Their description has been provided in previous papers [7, 8]. Glass particles are quickly coated with a thin calcium phosphate-rich layer containing traces of magnesium. The dissolution and reaction kinetics appear much faster for B75 glass particles compared to B67.5 glass particles. However, after a few days SiO_2–CaO–P_2O_5 particles are entirely changed into calcium phosphates, whereas SiO_2–CaO particles exhibit a different behavior: the previously Ca-P enriched periphery has been dissolved and glass particles consist of a silicate network. In order to quantify the changes in the glass matrix composition, elemental maps were divided in various regions of interest depend-

ing on the distribution of chemical elements. Thanks to Supavisio analysis software [9], we created thin masks of measurement at the periphery of the glass particles, in areas where the Ca-P enriched layer developed. With this methodology, calculation of elemental concentrations was made possible at the periphery of the grains. Results are presented in Figure 1. Each point corresponds to the average of concentrations calculated in several regions of interest. These regions of interest were defined over various samples in order to be ensured of measurements reproducibility.

During the first 15 minutes of interaction between B75 particles and biological fluids, Ca and P concentrations increase (Figure 1), as a result of the fast formation of a calcium phosphate layer at the periphery of the material. Si concentration decreases consequently in this region, as Si, Ca and P oxides concentrations represent nearly 100 % of the glass matrix. An important diminution in Ca concentration occurs at the periphery of B75 particles after 30 minutes soaking, because growing quantities of Ca are dissolved in consequence of the dealkalinisation of the glass surface. Therefore important changes in P and Si concentrations are observed at this time. Then, growth of the Ca-P layer continues at the periphery of the particles. Ions coming both from the glass matrix and biological fluids are incorporated at the surface of the particles. This is why a rapid increase in Ca and P concentrations and a decrease in Si concentration are observed until 6 hours of interaction. After 6 hours soaking, Ca concentration represents 33.4 % of the peripheral layer. The layer contains 9.1 % of P and 12.9 % of Si are still present. Traces of Mg are detected in the order of 0.9 %. However it seems clear that the newly formed Ca-P layer is unstable and that it is quickly dissolved by biological fluids; indeed, Ca and P concentrations drop to low values beyond 6 hours of interaction. In the meantime, Si concentration increases up to nearly 40 %. After 4 days of interaction with biological fluids, the periphery of B75 particles contains 42.3 % Si and 3.8 % Ca. Neither P nor Mg remains at the surface of the B75particles.

In comparison, the dissolution of the material and the formation of the Ca-P layer begin later for the P-containing B67.5 particles. After 1 h soaking, only little changes have occurred in the composition of the B67.5 particles periphery. It is only after 6 hours of interaction with biological fluids that significant changes are observed. Ca and P concentration then increase continuously with time of immersion in biological fluids so that the periphery of B67.5 particles is composed after 4 days of 34.3 % Ca and 15.6 % P. Important information are the presence of appreciable amount of Mg at the periphery of B67.5 particles: after 4 days of interaction Mg is incorporated in the Ca-P layer in the order of 0.6 %. Regarding Si concentration, there is evidence that the silicate network is progressively broken down at the periphery of the particles: after 2 days soaking, quantities of Si lower than 4 % remain in the peripheral regions.

In addition, elemental concentrations in the core of B75 and B67.5 glass particles were calculated for the different times of interaction. Figure 2 shows the results. During the first times of interaction, migration and diffusion of ions from the inner of the particles to their periphery lead to the observed fluctuations in the composition of the glass matrix. Then B75 and B67.5 particles exhibit opposite behaviors. B67.5 particles are progressively entirely changed into calcium phosphates. After 4 days of interaction, Si concentration in the core of B67.5 particles has deeply decreased and is equal to 14.2 %: the original silicate network has been gradually removed, giving way to a Ca-P-enriched core (27.2 % of Ca, 12.3 % of P) that contains traces of Mg. For B75 particles, the observations are totally different: the core of B75 particles grow poorer in Ca and P as the time of interaction with DMEM increases. As a consequence, increasing quantities of Si are detected. After 4 days of interaction, the composition of the core of B75 particles is close to that of the periphery: great quantities of Si are present to a total value of 40.2 wt %, Ca concentration is detected at a low value of 6.1 wt %.

Discussion

Different reaction stages are involved in the bioactivity process and have been identified for several years. Briefly, the alkaline and alkaline-earth ions present at the surface of the glass are first dissolved and leached in the solution. Then polycondensation reactions of surface silanols create a high-surface area silica gel that further provides a large number of sites for the formation and growth of calcium phosphates. Those latter will progressively crystallize into a biologically reactive hydroxycarbonate apatite equivalent to the mineral phase of bone [10, 11]. Concerning B75 glass particles, dealkalinisation of the glass matrix and ionic exchanges start very quickly, so that a calcium phosphate-rich layer is formed within minutes at the periphery of the particles. Such a high reactivity is directly linked to the high content of Si and Ca oxides in the glass matrix. In contact with an aqueous medium, Ca is a very soluble alkaline-earth element. SiO_2 represents 75 wt% of the glass composition; the higher the Si content, the thicker the hydrated, porous silica-gel layer formed after surface silanols polycondensation [12]. This generates a large active surface area which speeds up both the dissolution process and the ionic exchanges. Though the Ca-P rich layer is quickly formed, our measurements indicate that it is almost completely dissolved after a few days of interaction. The core of the particle, consisting of the enduring silicate network, solely remains.

Concerning B67.5 particles, the surface reactions involved in the bioactivity mechanism begin later. Nevertheless, after 4 days soaking the Ca-P-Mg layer has extended to the whole material: the composition of the core of B67.5 particles is close to that of the periphery, although greater quantities of Si still remain in the core of the material. These observations may be explained by the lower Si content in the primary glass; thus B67,5 particles can not have the advantage of an accelerated dissolution process such as for B75 particles. However, the Ca-P layer once formed, its growth and evolution are faster for B67.5 particles because of the presence of P in the initial glass matrix.

An essential observation is the final dissolution of the Ca-P peripheral layer after a few days of interaction for B75 particles, whereas the layer still remains for B67.5 particles. It provides us with an indication on the formation of hydroxyapatite at the surface of the particles. In fact, hydroxyapatite is the most stable and least soluble of all calcium phosphates [13]. Thus our results suggest that the periphery of SiO_2–CaO glass particles consists of amorphous calcium phosphates which composition significantly differs from that of hydroxyapatite. Indeed amorphous calcium phosphates are usually the first phase precipitated from a supersaturated solution and they are a transient phase during the formation of thermodynamically more stable hydroxyapatite [14]. The absence of P in the initial glass matrix may explain that SiO_2–CaO glass particles encounter great difficulties to achieve the transformation of their peripheral amorphous Ca-P layer into a more stable apatitic phase.

To better understand the B75 and B67.5 opposite behaviors, we calculated the Ca/P atomic ratios at the surface of the glass particles by creating thin masks of measurement about 1 μm thick at the glass/biological fluids interface. Results are presented in Table 1. They shall be compared to the 1.67 nominal value of stoechiometric hydroxyapatite. The Ca/P atomic ratio decreases as the time of interaction increases. For B75 particles, the lowest value is equal to 2.5 and is reached after 6 h of interaction. That is quite distant from the value of stoechiometric apatite. Beyond 6 h, the Ca/P ratio is upper than 15 because of the dissolution of the amorphous Ca-P layer, and the Ca/P ratio is thus not properly defined. For B67.5 particles, a low value of 1.6 is finally reached after 4 days soaking, which is very close to the 1.67 value of apatite. As a conclusion, an enduring apatitic phase seems to be formed at the periphery of SiO_2–CaO–P_2O_5 glass particles. Presence of phosphorus in the glass matrix facilitates the transformation of the initially amorphous calcium phosphates into apatite crystals. Initially limited to some scattered sites, the apatite layer then quickly extends on great depths because

the crystallized calcium phosphates could act as nucleation agents, increasing the kinetics of new layer formation [15].

Conclusion

A major advantage of PIXE-RBS nuclear microprobes is to enable accurate quantitative analyses of the glass particles/biological fluids interface. Indeed, optimizing parameters such as the composition and the texture of the glass, which are determinant for the material bioactivity, requires the collection of reliable quantitative information regarding the physico-chemical reactions occurring at the surface of the glass. Aiming at this, we highlighted the influence of phosphorus on the *in vitro* bioactivity of SiO_2–CaO–P_2O_5 glasses. Phosphorus-free B75 particles beneficiate from some favourable conditions that speed up both the dissolution and the initial formation of the Ca-P-Mg layer. Ionic exchanges are operated since the very first time of interaction with biological fluids. But contrary to the phosphorus-containing B67.5 particles, B75 particles only own a reservoir of Ca ions into the glass matrix: this is why B75 particles encounter great difficulties to transform their peripheral Ca-P layer into a more stable apatite-like phase. For SiO_2–CaO–P_2O_5 B67.5 particles, the Ca-P-Mg layer is extended on a great depth. Moreover calculation of the Ca/P atomic ratios at the glass/biological fluids interface indicates that the Ca-P-Mg layer is quickly changed into an apatitic phase. It might result in an improved chemical bond with living tissues.

Acknowledgement

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References

[1] L.L. Hench, J. Biomed. Mater. Res. 41 (1998) 511.

[2] L.L. Hench, J.M. Polak, Science 295 (2002) 1014.

[3] L.L. Hench, R.J. Splinter, W.C. Allen, T.K. Greenlee, J. Biomed. Mater. Res. Symp. 2 (1971) 117.

[4] L.L. Hench, J.K. West, Chem. Rev. 90 (1990) 33.

[5] J.A. Maxwell, W.J. Teesdale, J.L. Campbell, Nucl. Instr. Meth. B 95 (1995) 407.

[6] M. Mayer, Nucl. Instr. Meth. B 194 (2002) 177.

[7] J. Lao, J.M. Nedelec, Ph. Moretto, E. Jallot, Nucl. Instr. Meth. B 261 (2007) 488.

[8] J. Lao, J.M. Nedelec, Ph. Moretto, E. Jallot, Surf. Int. Anal., *in press* (2007).

[9] Y. Barbotteau, Available from http://biopixe.free.fr.

[10] L.L. Hench, J. Wilson, in: L.L. Hench and J. Wilson (Ed.), Introduction to bioceramics, World Scientific, 1993.

[11] K. Ohura, T. Nakamura, T. Yamamuro, T. Kokubo, Y. Ebisawa, Y. Kotoura, M. Oka, J. Biomed. Mater. Res. 25 (1991) 357.

[12] F. Devreux, P. Barboux, J. Nucl. Mater. 298 (2001) 145.

[13] J. Coreno, R. Rodriguez, M.A. Araiza, V.M. Castano, J. Mater. Chem. 8 (1998) 2807.

[14] S.V. Dorozhkin, M. Epple, Angew. Chem. Int. Ed. 41 (2002) 3130.

[15] M. Vallet-Regí, I. Izquierdo-Barba, A.J. Salinas, J. Biomed. Mater. Res. 46 (1999) 560.

Figure captions

Figure 1: Evolution of elemental concentrations at the periphery of B75 and B67.5 particles with time of exposure to biological fluids. Inset: evolution of elemental concentrations at the periphery of B75 particles during the first 6 hours of interaction.

Figure 2: Evolution of elemental concentrations in the core of B75 and B67.5 particles with time of exposure to biological fluids. Inset: evolution of elemental concentrations in the core of B75 particles during the first 6 hours of interaction.

Figures

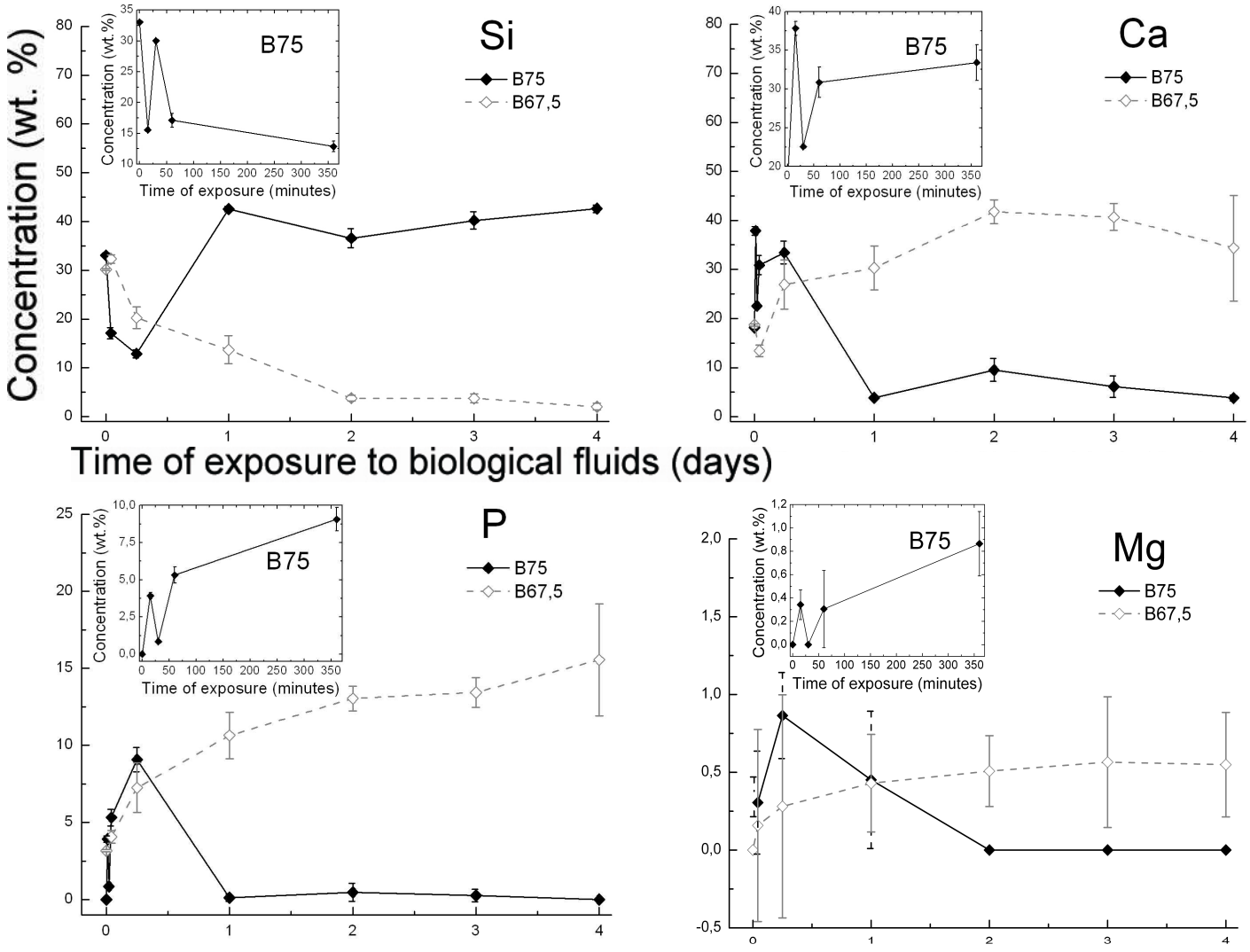

Figure 1

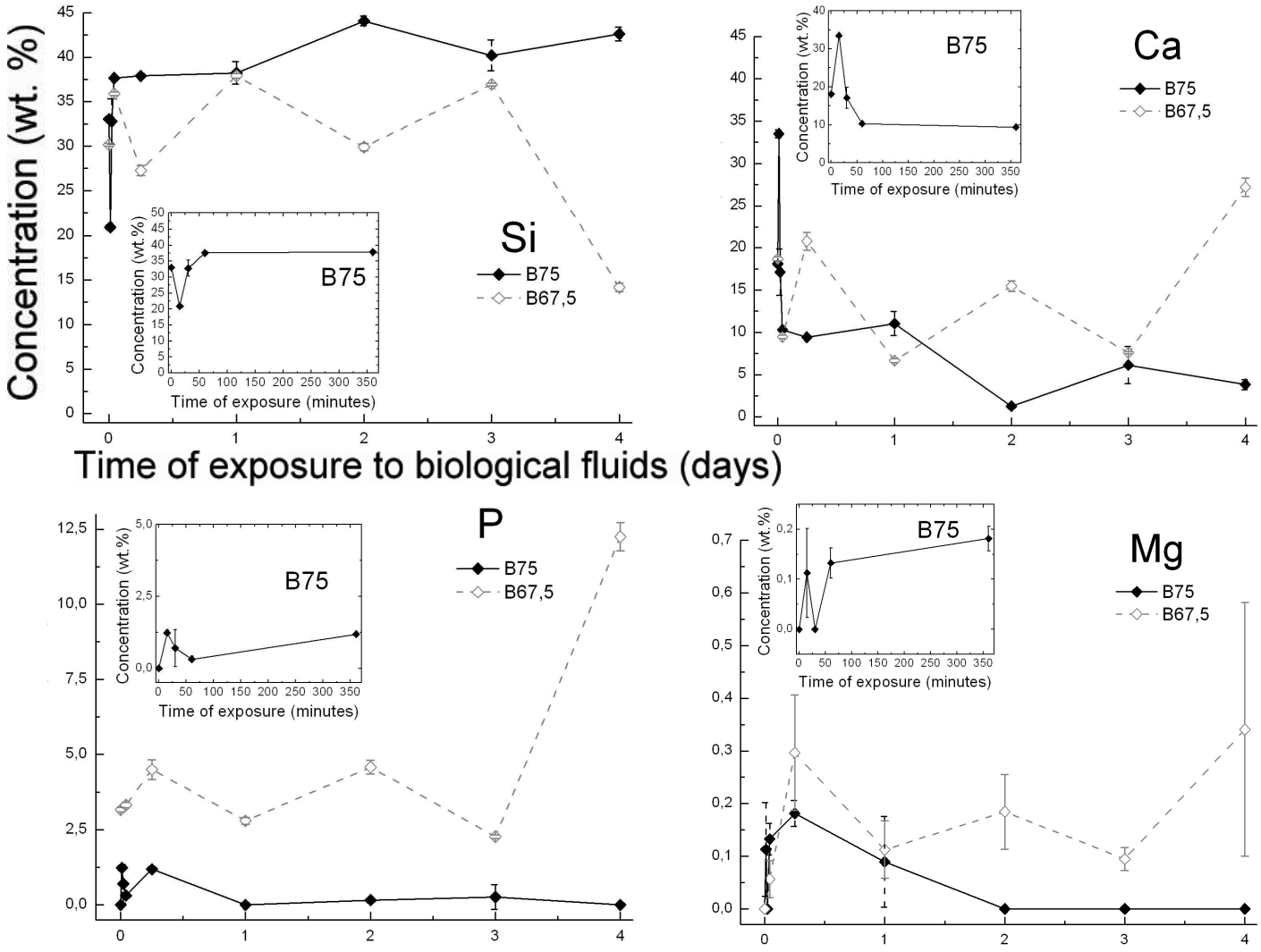
Concentration (wt. %)
Si
B75
B67,5
Concentration (wt.%)
Time of exposure (minutes)
Ca
P
Mg
Time of exposure to biological fluids (days)

Figure 2

Tables

	1h	6h	1 d	2 d	3 d	4 d
B75	3.8	2.5	N/A	N/A	N/A	N/A
B67.5	2.6	2.8	2.1	2.4	2.3	1.6

Table 1 : evolution of Ca/P atomic ratios calculated at the surface of B75 and B67.5 particles with time of exposure to biological fluids. Beyond 6 hours of interaction, the Ca/P atomic ratio is considered to be not available (N/A) for B75 particles, because of the dissolution of the Ca-P layer.